Monoclonal Antibody Charge Heterogeneity Analysis by Capillary Isoelectric Focusing on the Agilent 7100 Capillary Electrophoresis System

Application Note

Biopharma

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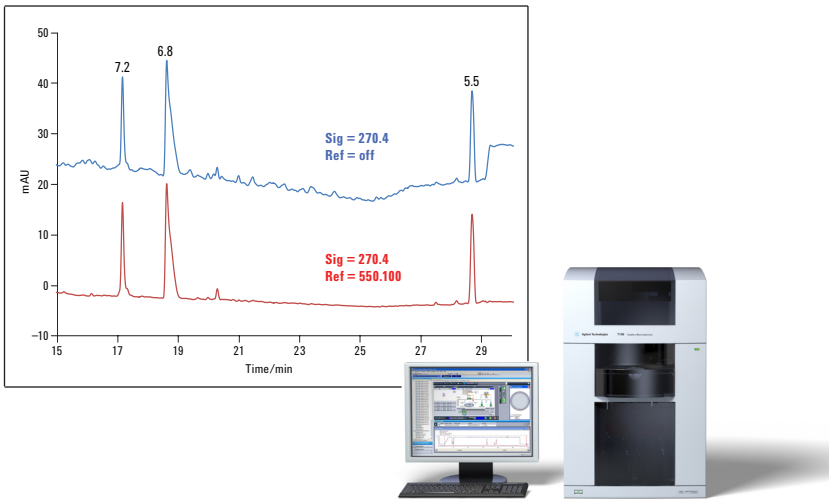

Abstract

Due to the importance of monoclonal antibodies (mAb) as therapeutic agents, there is a growing demand for high resolution analytical methods to characterize these complex molecules. One important property of an mAb is its charge state that may change during and after manufacturing. A very useful technique to measure mAb charge heterogeneity is capillary isoelectric focusing (cIEF). This Application Note shows how the Agilent 7100 CE system can be used for high resolution mAb charge isoform analysis applying cIEF.

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Introduction

Capillary electrophoresis (CE) methods have become indispensable tools for biopharmaceutical product development and quality control[1]. For identity and purity determination of proteins, such as monoclonal antibodies (mAb), several CE-based techniques are used: capillary gel electrophoresis (CGE), which separates according to hydrodynamic size and capillary zone electrophoresis (CZE) or capillary isoelectric focusing (cIEF), which addresses charge heterogeneity.

Capillary IEF offers several advantages compared to slab gel-based IEF techniques, such as increased automation, reproducibility, and quantitative analysis, and has therefore replaced these methods in many biotech laboratories. Modifications that can affect the charge state of a monoclonal antibody are amidation/deamidation, C-terminal lysine loss, formation of N-terminal pyroglutamate, or glycan sialylation. Since these modifications can have a profound impact on the immunogenicity and overall biological activity of the therapeutic product, monitoring of these charge variants is requested by regulatory agencies.

This Application Note describes the performance of a popular high resolution cIEF method for mAb charge isoform analysis[2,3] on the Agilent 7100 Capillary Electrophoresis system. The results of an intermediate precision study employing four different CE instruments are presented.

Experimental

Materials

IgG1 Kappa from murine myeloma clone number MOPC 21 (mIgG1-κ), IEF-markers, urea, L-arginine, iminodiacetic acid and tris(hydroxymethyl) aminomethane were obtained from Sigma Aldrich (St. Louis, MO, USA), Pharmalyte 5-8 from GE Healthcare Bio-Sciences AB (Uppsala, Sweden), hydrochloric acid and glacial acetic acid from Merck (Darmstadt, Germany) and phosphoric acid from JT Baker (Austin, TX, USA). A 0.05 × 670 mm neutral coated capillary (Beckman Coulter Part Number 477441) and cIEF gel (Beckman Coulter Part Number 477497) were obtained from Beckman Coulter (Fullerton, CA, USA). All other materials and instrumentation were from Agilent Technologies (Waldbronn, Germany).

Sample preparation

Prior to CE analysis, mAb samples were desalted using Microcon YM-30 centrifugal filter devices (Millipore, Bedford, MA, USA) and a buffer containing 20 mM Tris/HCl, pH 8. Protein concentrations were measured with the Qubit assay (Life Technologies, Paisley, UK) and were between 2–3 mg/mL after desalting. Sample solutions for cIEF analysis were prepared by adding the following reagents into 0.5 mL microcentrifuge vials:

- 100 µL of cIEF gel containing 3 M urea
- 3.0 µL of Pharmalyte 5-8
- 4.5 µL of 500 mM L-arginine (cathodic stabilizer)
- 5.0 µL of 200 mM iminodiacetic acid (anodic stabilizer)
- ~ 3 µL of IEF-marker mix
- 10 µL of desalted mAb

Final concentrations in the sample solution were 80% cIEF gel, 2.4 M urea, 2.4% Pharmalyte 5-8, 18 mM L-arginine, 8 mM iminodiacetic acid and 0.16–0.24 mg/mL mAb. Volumes used for IEF-markers 5.5/6.2/6.6/6.8/7.2 were 0.5/1.0/0.2/2.0/0.2 µL corresponding to final concentrations of 12/8/1.6/16/1.6 ng/mL. Mixtures were vortexed for 10 s, centrifuged briefly and transferred into 100 µL CE sample vials. Sample solutions were kept in the autosampler carousel of the CE instrument at about 10°C and analyzed within 24 h.

CE conditions

An Agilent 7100 CE system equipped with an external waterbath set to 6 °C, the detector filter assembly (p/n G7100-62700) and 4 bar external pressure were used for all CE runs. The neutral coated capillary was cut at both ends at a distance of 8.5 cm and 24.5 cm from the detection window, respectively, equipped with a green alignment interface (p/n G7100-60210) and fitted into the Agilent capillary cassette. Once a day, capillaries were conditioned as follows: high pressure flush at 3.5 bar with 350 mM acetic acid for 5 minutes, with water for 2 minutes and with cIEF gel for 5 minutes. Prior to every run, capillaries were conditioned as follows: high pressure flush at 3.5 bar with 4.3 M urea solution for 3 minutes and with water for 2 minutes. Samples were injected by applying 2 bar high pressure for 100 seconds, followed by a water dip of both inlet and outlet electrode. Focusing was done for 5 minutes at 25 kV with 200 mM phosphoric acid as anolyte and 300 mM NaOH as catholyte. For chemical mobilization, the oulet vial was exchanged for 350 mM acetic acid and 30 kV was applied for 30 minutes. After each run, a high pressure flush at 3.5 bar with water was done for 2 minutes. After capillary use, a high pressure flush at 3.5 bar with water for 2 minutes and with cIEF gel for 5 minutes was done and the capillary ends were placed in water filled vials. If it was not in use for 3 or more days, the cIEF gel filled capillary was stored with the ends submerged in water at 2-8 °C. All flushes were done in forward direction, i.e. pressure was applied to the inlet vial. The capillary temperature was kept at 20 °C. The detection wavelength was 270 nm with a bandwidth of 4 nm (no

reference wavelength) if not otherwise stated. The detector response time was 2 seconds. For all reagents, 2-mL glass vials were used. The fill volume was 1.6 mL, except for the waste vials that were empty. All reagent vials were exchanged after three runs.

Results and discussion

Method adaptation to the Agilent 7100 CE instrument

A commercially available mouse mAb was used as a model protein to show the suitability of the Agilent 7100 CE system for running a high resolution cIEF method[2,3] employing carrier ampholytes in the pH range 5–8. To suppress the electroendosmotic flow, the separation was done in a neutral coated capillary filled with a gel containing a mixture of ethylene glycol and poly(ethylene oxide) in water and in addition urea for increased protein solubility. Anodic stabilizer iminodiacetic acid and cathodic stabilizer L-arginine were added to the sample solution to avoid loss of carrier ampholytes and sample components during focusing (see Experimental).

Figure 1 presents results measured on a single instrument. In terms of peak height, analysis time, and resolution these results were very similar to published data obtained with the same sample[2]. Two method changes were implemented in order to adapt the high resolution cIEF technique to the 7100 CE system. First, a slightly longer capillary was used with a total length of 33 cm and an effective length of 24.5 cm. This was necessary to fit the capillary into the cassette of the 7100 CE system. To compensate for the increased runtime due to capillary length, the concentration of the anodic stabilizer iminodiacetic acid in the sample solution was increased from 4 to 8 mM. Second, adsorption was recorded at 270 nm. This detection wavelength was chosen because the light intensity available at 280 nm was limited due to the installed detector filter assembly[4]. This filter transmits light only around 260 nm and above 450 nm. It was used to protect sample compounds from denaturation by the high energy UV light. One drawback of the detection at a shorter wavelength is an increased background due to higher ampholyte absorption. However,

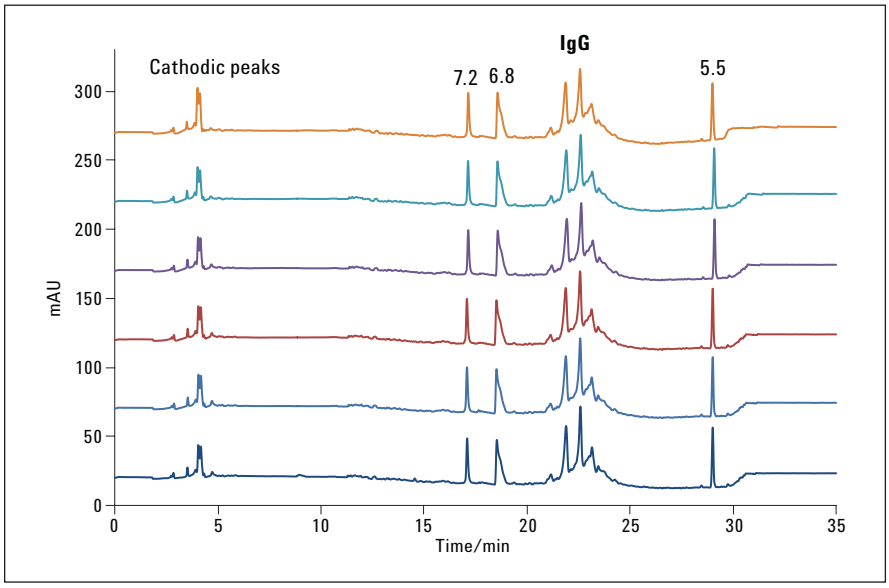

Figure 1
Monoclonal antibody isoform analysis on the Agilent 7100 CE system. A sample containing mIgG1-κ and IEF-markers 5.5, 6.8 and 7.2 was analyzed by high resolution cIEF. Shown are the electropherograms of six subsequent injections.

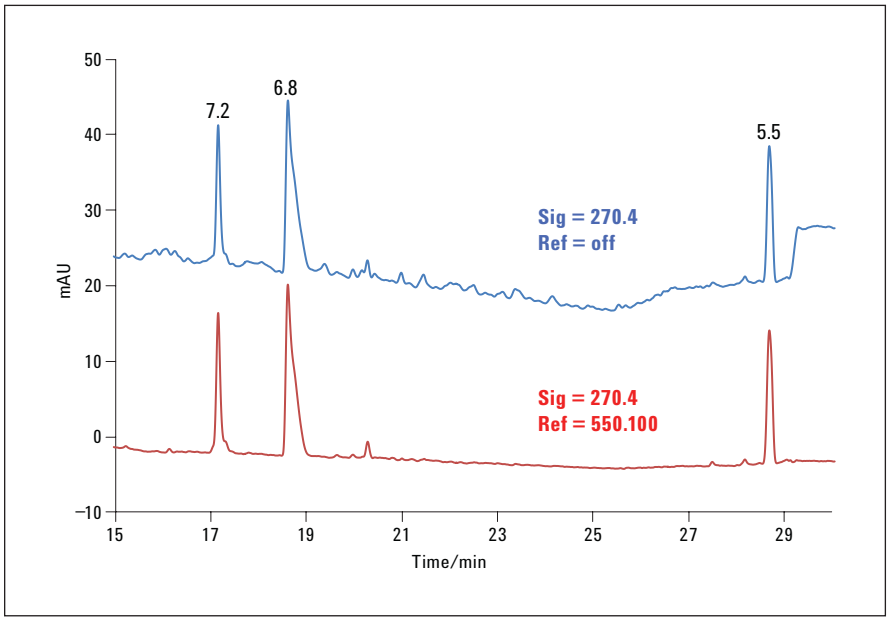

Figure 2
Data collection with and without reference wavelength. A sample containing IEF-markers only was analyzed by cIEF. Shown are electropherograms of the same run that were recorded without (blue) and with reference wavelength (red). The ASTM noise between 21 min and 25 min was 0.12 mAU without reference wavelength and 0.039 mAU with reference wavelength. The IEF-marker height was about the same in both cases.

this background could be reduced to about one third if a reference wavelength in the visible range was used (Figure 2). Another advantage of applying a reference wavelength was an improved baseline stability.

Intermediate precision

A quantitative analysis of mAb isoform isoelectric point (pI) and relative abundance was performed with the adapted cIEF method on four different instruments. Peaks were automatically integrated with the ChemStation software and mAb peaks were assigned to isoform groups as shown in Figure 3. Apparent isoelectric points were calculated by linear regression analysis of marker pI versus migration time. As shown in Table 1, the apparent pI intermediate precision was 0.105 RSD% or better for all isoforms. This agrees with published within-laboratory values of about 0.1 RSD%[3]. For relative peak area, the intermediate precision was good for main isoforms B, C and D that account for more than 20% of relative peak area each. However, for minor isoforms A and E values > 10 RSD% were observed (Table 1). These relatively high values might be explained by the less defined borders of these minor isoforms (Figure 3) and the background due to ampholyte absorption. Both effects in combination presented a challenge for the integration algorithm. However, all data presented in Figures 1, 3-4 and Table 1 were obtained without reference wavelength. Most probably the usage of a reference wavelength would have improved the result (Figure 2).

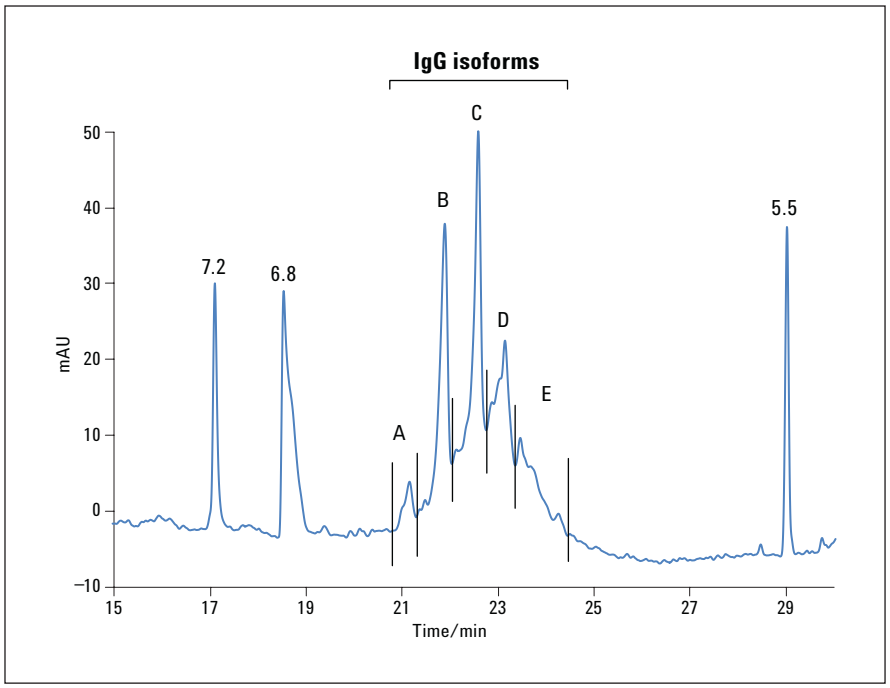

Figure 3
Monoclonal Ab isoform quantification. A part of an electropherogram of Figure 1 was enlarged to show the assignment of mIgG1-peaks to 5 isoform groups A-E as indicated.

Isoform group	Apparent pI			Peak area (%)		
	Average	SD	RSD%	Average	SD	RSD%
A	6.546	0.007	0.105	4.29	0.47	10.93
B	6.457	0.004	0.056	23.22	0.58	2.50
C	6.365	0.003	0.042	32.94	0.69	2.08
D	6.290	0.002	0.036	25.66	1.48	5.77
E	6.232	0.002	0.029	13.89	1.76	12.67

Table 1
Intermediate precision of high resolution cIEF on the Agilent 7100 CE system. Data were obtained on four CE instruments with six runs/instrument on four different days (n=24). For the assignment of peaks to isoform groups A-E refer to Figure 3.

Analysis of a set of different mAb

Three different mAb from biotech companies with isoelectric points within 5.5 and 6.8 were analyzed to show the applicability of the adapted cIEF method (Figure 4). The separation of charge isoforms was possible in every case. A particularly informative electropherogram was obtained for mAb2, in which six charge isoforms could be clearly separated.

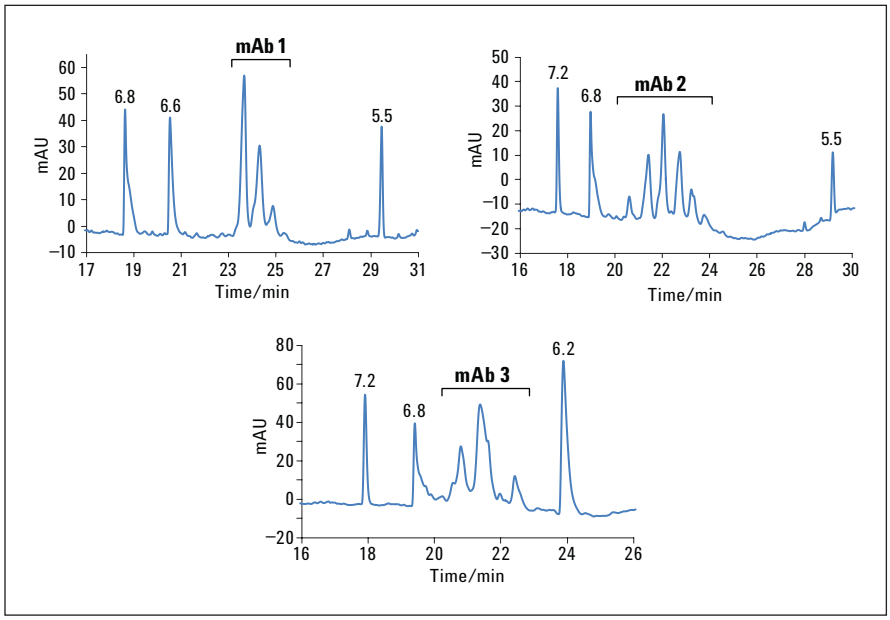

Figure 4
Analysis of a set of different mAb. Three mAb from biotech companies were analyzed with the same method as mIgG1-κ. For mAb1 and mAb3 different sets of IEF-markers were used.

Conclusion

This work shows that mAb charge heterogeneity analysis can be performed reliably and with high precision on the Agilent 7100 CE system. An established high resolution cIEF method was run with minor adaptations on this system and delivered results comparable to published data. Isoelectric points and the relative abundance of main mAb charge isoforms were determined with good intermediate precision. The applicability of the method was demonstrated for a set of different mAb. The flexible design of the 7100 CE system allows easy adaptation of an existing cIEF method. This ease-of-use supports fine-tuning methods for existing commercial kits in cIEF or CGE[5] as well as individual CZE method development. The air-cooled cassette provides quick exchange of any standard fused silica capillary and the diode array detector of the 7100 CE system supports optimization of methods for analytical sensitivity in a wide range of applications.

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